Reversal of Allergen-induced Airway Remodeling by $CysLT_1$ Receptor Blockade

William R. Henderson, Jr., Gertrude K. S. Chiang, Ying-tzang Tien, and Emil Y. Chi

ONLINE DATA SUPPLEMENT

METHODS

Animal study protocol. All animal use procedures were approved by the Animal Care Committee of the University of Washington; mice were anesthetized with 0.2-0.3 ml intraperitoneal ketamine (6.5 mg/ml)/xylazine (0.44 mg/ml). Female BALB/c mice (6-8 wk of age; The Jackson Laboratory, Bar Harbor, ME) were employed. The OVA-treated groups received intraperitoneal injections of 100 μg OVA (0.2 ml of 0.5 mg/ml) complexed with alum on Days 0 and 14 and intranasal doses of 100 μg OVA (0.05 ml of 2 mg/ml) on Day 14 and 50 μg OVA (0.05 ml of 1 mg/ml) on Days (± 1-2 days) 25, 27, 29, 46, 60, 69, 71, and 73. On Day 73 (30 min after OVA challenge), groups of mice had subcutaneous placement of mini-osmotic pumps (200 μl Alzet Model 2004 osmotic pumps; 6 μl/d delivery rate; Durect Corporation, Cupertino, CA) containing the $CysLT_1$ receptor antagonist montelukast sodium (1 mg/kg), the corticosteroid dexamethasone (1 mg/kg; dexamethasone-water soluble, Sigma Chemical Co., St. Louis, MO), montelukast (1 mg/kg) + dexamethasone (1 mg/kg), or saline control (4-8 mice per study group) with pump replacement on Days 103 and 133. The control group received intraperitoneal normal saline with alum on Days 0 and 14 and intranasal saline without alum on Days (± 1-2 days) 14, 25, 27, 29, 46, 60, 69, 71, and 73.

The 1 mg/kg dexamethasone dose was determined in preliminary studies (see Results) to significantly block allergen-induced eosinophil trafficking to the airways in an acute asthma model. Groups (n=5 each) of OVA-sensitized mice given intraperitoneal OVA with alum on Days 0 and 14 and intranasal OVA on Days 14, 25, 27, and 29 as described above received either dexamethasone 1 mg/kg or saline intraperitoneally 30 min prior to each intranasal OVA challenge on Days 25, 27, and 29. Bronchoalveolar lavage (BAL) was performed on Day 30, 24 hours after the last OVA administration in this acute asthma model. The control group received intraperitoneal saline with alum on Days 0 and 14 and intranasal saline without alum on Days 14, 25, 27, and 29.

Pulmonary function testing. Noninvasive pulmonary mechanics were determined on Days 73 (30 min after OVA) and 163 (three months after the last OVA challenge) after aerosolization of methacholine in conscious, freely moving, spontaneously breathing mice using whole body plethysmography (Model PLY 3211; Buxco Electronics Inc., Sharon, CT) (E1, E2). The degree of bronchoconstriction was expressed as enhanced pause (Penh), a calculated dimensionless value that correlates with measurements of airway resistance, impedance, and intrapleural pressure. P_{enh} readings were taken and averaged for 4 min after each nebulization challenge. P_{enh} was calculated as follows: $P_{enh} = [(T_e/T_r-1) \times (PEF/PIF)]$, where T_e is expiration time, T_r is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow x 0.67 coefficient. The time for the box pressure to change from a maximum to a user-defined percentage of the maximum represents the relaxation time. The T_r measurement begins at the maximum box pressure and ends at 40%.

BAL. After completion of plethysmography on Days 73 and 163, mice were exsanguinated by cardiac puncture and the left lung isolated by tying off the left main stem bronchus (E3). The right lung was lavaged with one wash of 1 ml of saline and then additional lavage of 1 ml in and out five times for a total of 2 ml. The total number of leukocytes per 0.05 ml aliquot was determined after methylene blue nuclear staining. The remaining BAL fluid was centrifuged at 200*g* for 10 min at 4°C, and the cell pellets were resuspended in saline containing 10% BSA with smears made on glass slides. Eosinophils were determined by staining with 0.05% aqueous eosin with methylene blue counterstaining (E3).

Lung histology and morphometry. The left lung [mouse lung anatomy is illustrated in (E4, E5, E6)] was cut horizontally into thirds with the upper two pieces fixed in 4% phosphate-buffered formalin at 20ºC for 15 h (E3). After embedding in paraffin, the tissues were cut into 5-μm sections. Two 5-μm sections were placed on each glass slide with 10 slides made for each block. For each type of morphometric analysis described below, 10 different airways (~0.4-0.7 mm in diameter and surrounded by smooth muscle cells) per mouse were randomly selected and examined by individuals blinded to the protocol design (E1, E7, E8).

The lung sections were stained with hematoxylin and eosin to determine the airway inflammatory cell infiltration on a semi-quantative 0-4+ scale and eosinophil number per unit lung tissue area (2,200 μm^2) (E3). Each light microscopic field at magnification x400, using a Whipple micrometer disc (Bausch & Lomb, Rochester, NY), represented 2,200 μm^2 of tissue area. The smooth muscle mass was determined as the thickness (μm) of the smooth muscle cell layer beneath the airway epithelial cell basement membrane measured at three sites tangential to each airway cross-section examined (E1). The sections were stained with alcian blue, pH 2.5, with nuclear fast red counterstaining to identify goblet cells (E3). The number of goblet cells was determined as percent of total airway epithelial cells in each airway examined (E3). To determine subepithelial fibrosis in the lungs, the sections were stained with Masson's trichrome stain (E1). The intensity of the collagen deposition (0-4+ semi-quantitative scale) was assessed in the immediate region beneath the epithelium containing the contractile elements and ECM associated with the airways as described by Leigh et al. (E9).

Immunocytochemistry. For immunocytochemistry using light microscopy (E10), mouse lung tissue was fixed in methyl Carnoy's solution, embedded in paraffin, and 5 μm sections cut, placed on Super Frost Plus slides (VWR Scientific, West Chester, PA), deparaffinised, hydrated, and rinsed in phosphate-buffered saline (PBS), pH 7.4 (Sigma Chemical Co.). All immunolabeling procedures were performed at 24°C. Endogenous peroxidase was inactivated by incubating the tissue sections in 0.75% hydrogen peroxide for 30 min. After rinsing in PBS, the sections were incubated with the primary antibody, polyclonal goat anti-CysLT$_1$ receptor-specific antisera (provided by Jilly F. Evans, Merck & Co., Inc.) at a 1:80 dilution for 25 min followed by rinsing in PBS and incubation with the secondary antibody, rabbit anti-goat antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA) at a 1:50 dilution for 25 min. Two controls were employed substituting the goat anti-CysLT$_1$ receptor primary antibody incubation with either PBS or normal goat IgG (Vector Laboratories). After rinsing in PBS, the sections were incubated with 0.5% 3’,3’-diaminobenzidine tetrachloride (Sigma Chemical Co.) in PBS and 0.15% hydrogen peroxide for 15 min for detection of peroxidase. The sections were rinsed in PBS and nuclei counterstained with 1% methyl green in distilled water for 3 min. The sections were dehydrated in ethanol, cleared in xylene, and mounted on glass slides with Permount (Fisher Scientific, Pittsburgh, PA).

Statistical analysis. The data are reported as the mean ± SE of the combined experiments. Differences were analyzed for significance ($p<0.05$) by analysis of variance (ANOVA) using the protected least significance difference method.

REFERENCES

E1. Henderson WR, Jr., Tang L-O, Chu S-J, Tsao S-M, Chiang GKS, Jones F, Jonas M, Pae C, Wang H, Chi EY. A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. *Am J Respir Crit Care Med* 2002;165:108-116.

E2. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, Gelfand EW. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156:766-775.

E3. Henderson WR, Jr., Lewis DB, Albert RK, Zhang Y, Lamm WJE, Chiang GKS, Jones F, Eriksen P, Tien Y, Jonas M, Chi EY. The importance of leukotrienes in airway inflammation in a mouse model of asthma. *J Exp Med* 1996;184:1483-1494.

E4. Gomes RF, Bates JH. Geometric determinants of airway resistance in two isomorphic rodent species. *Respir Physiol Neurobiol* 2002; 130:317-325.

E5. Reader JR, Tepper JS, Schelegle ES, Aldrich MC, Putney LF, Pfeiffer JW, Hyde DM. Pathogenesis of mucous cell metaplasia in a murine asthma model. *Am J Pathol* 2003; 162:2069-2078.

E6. Baker GL, Shultz MA, Fanucchi MV, Morin DM, Buckpitt AR, Plopper CG. Assessing gene expression in lung subcompartments utilizing in situ RNA preservation. *Toxicol Sci* 2004;135-141

E7. Bertram JF, Bolender RP. Counting parenchymal cells in the goat lung with serial section reconstruction and stereology. *Am Rev Respir Dis* 1986;77:133:891-898.

E8. Bolender RP, Hyde DM, Dehoff RT. Lung morphometry: a new generation of tools and experiments for organ, tissue, cell, and molecular biology. *Am J Physiol* 1993;265:L521-L548.

E9. Leigh R, Ellis R, Wattie J, Southam DS, De Hoogh M, Gauldie J, O'Byrne PM, Inman MD. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* 2002;27:526-535.

E10. Henderson WR, Jr., Chi EY, Albert RK, Chu S-J, Lamm WJE, Rochon Y, Christie PE, Harlan JM. Blockade of CD49d (α_4 integrin) on intrapulmonary but not circulating leukocytes inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma. *J Clin Invest* 1997;100:3083-3092.

FIGURE LEGENDS

Figure E1. Effect of $CysLT_1$ receptor blockade and corticosteroid treatment on allergen-induced increased airway smooth muscle mass and collagen deposition. Lung tissue was obtained on Day 163 from saline controls (*A*) and OVA-sensitized/challenged mice in the absence (*B*) or presence of montelukast (*C*), dexamethasone (*D*), or montelukast + dexamethasone (*E*), and stained with Masson's trichrome stain. *AW*, Airway. Bars = 100 μm.

Figure E1.

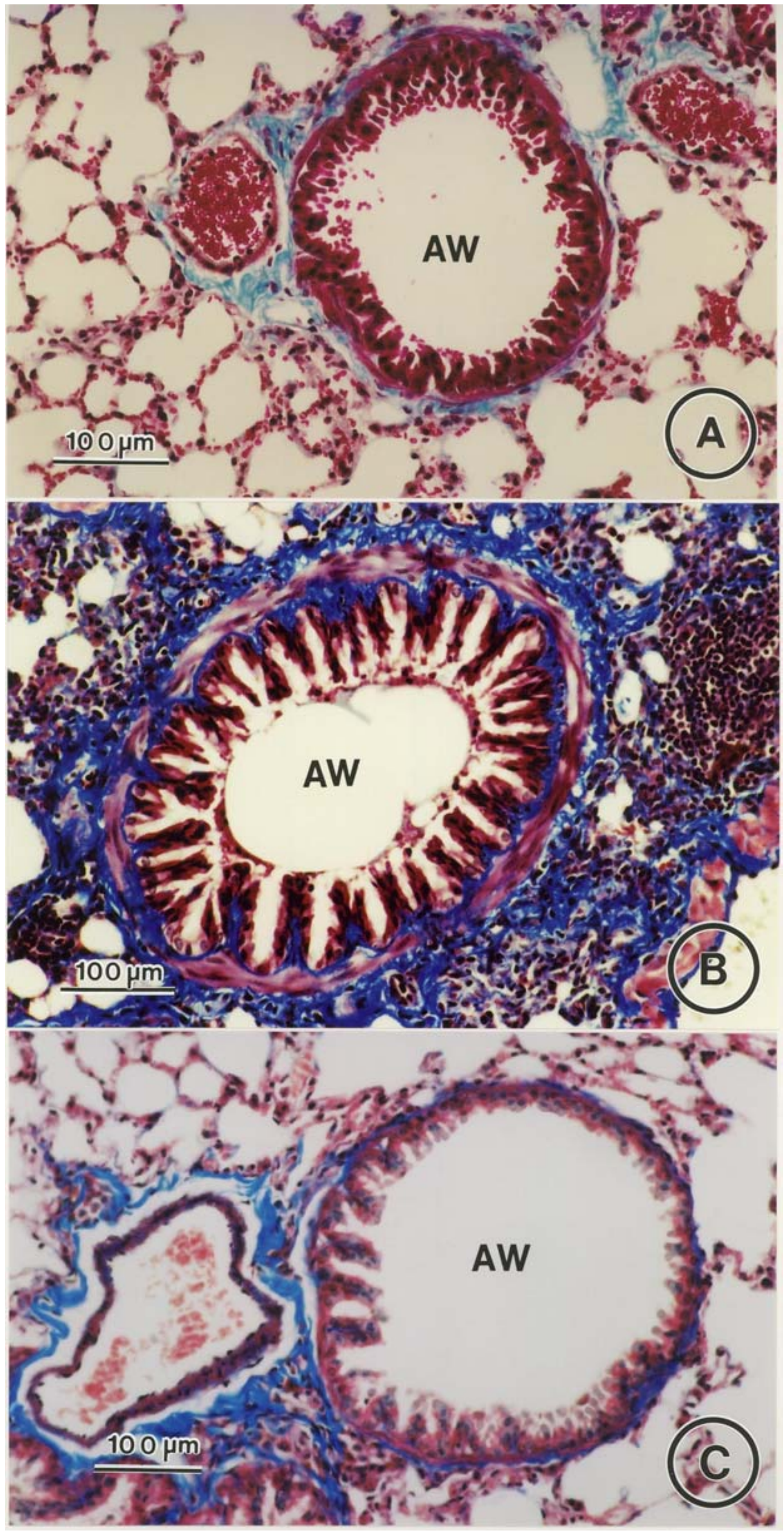

Figure E1 (Cont.)